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The Accuracy of API 20E and PCR Using 16s rRNA Gene for Characterization of *Escherichia coli* Strains Causing Urinary Tract Infections in Damascus-Syria

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Abstract: Urinary tract infections (UTIs) are one of the most widespread diseases in developing countries including Syria. The current study aims to evaluate the efficacy of the different methods: EMB agar, API 20E and polymerase chain reaction (PCR), in identifying a collection of *Escherichia coli* (*E. coli*) isolates that cause UTIs. In This study, were diagnosed isolates taken from samples of patients with UTIs. These isolates were obtained in the form of colonies identified as *E coli* by conventional methods on EMB agar. The colonies were identified using API 20E kit. Further confirmation was performed by PCR amplifying a target fragment of the *E. coli* 16S rRNA gene. Only 51 of the 75 isolates identified as *E. coli* using EMB agar indicated a positive results as *E. coli* after applying biochemical tests. Therefore, the error rate is 32%. After the application of PCR, we founded 65 isolates were *E. coli* which means the error rate is 13%. The PCR method showed good agreement with the conventional method (86.6%). We observed significantly the reduction of error rate when using PCR method comparing with biochemical method. This confirmed the inaccuracy of *E. coli* identification based on her formal characteristics and the biochemical testes only.
Keywords: Urinary Tract Infections; polymerase chain reaction (PCR); *Escherichia coli*; Diagnosis.

Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases encountered in the clinical practice, mainly being associated with different members of the *Enterobacteriaceae* family[3,17]. This disease is acquired from hospitals (Nosocomial Infection) 46 % rate, also acquired from the community and constitute 80 % of cases of chronic prostatitis and 90 % of cases of pyelonephritis[10] also includes inflammation of the bladder and urethra[21]. It is the second most frequent diseases between human groups[18]. It is estimated that between 40 and 50% of women experience a UTI at least once during their lifetime, and that 33% of women in the United States suffering from a UTI require antimicrobial therapy by the age of 24[7,8]. UTIs are also a common cause of febrile illness in young children. It has been estimated that UTIs are diagnosed in 1% of boys and in 3–8% of girls[22]. And we choose UTIs for study because are one of the most common infectious diseases in humans and are a major cause of morbidity and mortality[14]. There are many known microbial causes of UTIs, including *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus* and *Escherichia coli*[14]. In fact, *E. coli* are the most predominant pathogen causing UTIs[25]. *E. coli*, the most prevalent facultative gram-negative bacillus in the human fecal flora, usually inhabits the colon as an innocuous commensal[12].

Extraintestinal pathogenic *E.coli* (ExPEC) strains causing UTIs are called uropathogenic *E. coli* (UPECs) are responsible for between 70 and 90 % of community-acquired UTIs and approximately 40 % of all nosocomial UTIs[28]. *E.coli* was selected for several reasons: 1) these germs are known and available frequently in the laboratory in Damascus; 2) And it is easy diagnosis on agar transplantation as EMB agar; 3) and sometimes causes serious lesions both in the urine or blood infections or vital fluids in Damascus.

Different commercial miniaturized systems were developed to identify presumptive

E. coli isolates of clinical origins. These identification systems include the widely used API 20E (bioMerieux, France) and Eosin Methylene Blue agar (EMB). The API 20E is one of the original miniaturized systems; still in wide spread use for the identification of members of the *Enterobacteriaceae* since the 1970s. This system is based on 23 different biochemical tests such as the production of indole; citrate utilization; voges-proskauer reaction; carbohydrate fermentation and other tests [27].

In the study of Hussein H. Abulreesh (2014), PCR was used to confirm the accuracy of commercial systems by randomly selecting isolates. The primers used were targeting a fragment of the *E. coli* 16S rRNA gene. The results showed that PCR further confirm the identification of the isolates. In general the results reported in his study with PCR reaffirm that PCR provide sensitivity in the identification of *E. coli* when compared with commercial methods. Perhaps a combination of two methods might be appropriate. However in routine laboratories this might be laborious, and therefore PCR might be used to further confirm the result of rapid identification systems [1].

In the current study were identified isolates of urine samples taken from patients with UTIs from different age groups of females and males in traditional method and biochemical using API 20E kit for identification of bacterial strains of the family Enterobacteriaceae, and molecular method applying PCR based on 16s rRNA gene.

Materials and methods

Bacterial isolates.

A collection of 75 Isolates from urine sample for adults and children patients with UTIs in deferent age groups, males and females, were collected in the form of bacterial colonies on the EMB agar. These Samples were taken from tow general hospitals in Damascus and during a period of time ranging between 7/2012 and 8/2013. All ethical issues were considered and this research was performed with hospitals' permission. The name and characters, personal information and even patients' illnesses and their medical information remained secret. All 75 isolates were purified on EMB agar plates (Titan Biotech- India). Incubation was at 37□ C for 24 hours. On EMB if *E. coli* is grown it will give a distinctive metallic green sheen (colonies with 2-3 diameter, exhibiting a greenish metallic sheen by reflected light and dark purple centers by transmitted light due to the metachromatic properties of the dyes), some species of *Citrobacter* and *Enterobacter* will also react this way to EMB. We transplant all isolates on EMB agar for purification and application of comparison.

Biochemical tests set (API20E kit).

Before the start of identification tests, all 75 isolates were purified on EMB agar plates to ensure that there is no mixed culture that could influence the reactions of the identification systems. A bacterial suspension approximating a 0.5 McFarland standard was prepared from each of the 75 purified isolates and was used for inoculation of the API 20E strips (bioMerieux, France); incubation was at 37 °C for 24 hours, after incubation the addition of reagents and interpretation of reactions were done according to manufacturers' directions. We have selected the API 20E kit, a standardized identification system for *Enterobacteriaceae* family and other non-fastidious, Gram-negative rods, which tested 23 biochemical differential characteristics. These characteristics are:

- **Investigation the effectiveness of enzymes**: galactoberanusidaz, arginine dihedrollaz, oxidase.
- **Interactions of decarboxylation group of amino acids**: lysine, ornithine.
- **Interactions of Deamination** of the amino acid tryptophan.
- **Fermentation reactions of following sugars**: glucose, mannitol, inositol, sorbitol, rhamnose, glucose, Mlebeoz, amygdalin, arabinose.
- **Interactions of productio**n: indole, asetoin.

- **Interactions of hydrogen sulfate gases produced** H2S, nitrogen dioxide NO2, nitrogen N2.
- **Study of the interactions:** gelatin hydrolysis, using citrate, urea hydrolysis (urease).

The 23 biochemical test reactions were converted into an octal profile number. Each profile number was then decoded by using the Analytical Profile Index for the completion of identification.

Isolation of Bacterial genomic DNA.

Each strains of *E. coli* was cultured in 3 ml LB broth medium (Luria-Bertani medium), (Titan Biotech-India) overnight at 37°C with aeration. Genomic DNA was isolated from the bacterial pellet after centrifugation of 1 ml of medium using Wizard DNA purification kit (Promega USA) according to the manufacturer's instructions. Purified DNA was pooled and quantified by spectrophotometry (JENWAY- Genova England); an A260/A280 ratio of 2 was found, confirming the purity of the DNA sample.

Identification by PCR Detecting Fragment of the E. coli 16S rRNA Gene

Identification by PCR was carried out on all selected 75 isolates. These isolates were identified as *E. coli* by EMB agar. And some of these isolates were identified as

E. coli by API 20E (51 isolates). The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rRNA. Primers flank hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria[13, 23]. The sequences of the PCR primers were designed as follows: wl-3110 (F) AGAGTTTGATCMTGGCTCAG, and wl-3111 (R) CCGTCAATTCATTTG AGTTT[15, 29]. These primers generate the 919 bp PCR fragment that is characteristic for all *E. coli* strains. Each PCR reaction was performed using 25 ìl of reaction mixture (PCR master mix Thermo scientific UK) consisting of 100–200 ng of DNA, 12.5 ìl of 1× dream taq buffer, and 0.4 mM for each (dATP, dCTP, dGTP, dTTP)and 4 mM Mgcl2. 12.5 ìl of water nuclease free. All of the reagents were thawed completely on ice and all of the reactions were assembled on ice. The reaction parameters were 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 5 min. In order to reduce non-specific amplification, the thermal cycler was pre-warmed to 80 °C before the reaction tubes were inserted. PCR amplification of the DNA was confirmed by running 10 ìl of the PCR product on a 1.5% agarose gel (ß-Mupid, Mini Agarose Gel Electrophoresis USA). Gel images have been captured digitally and analyzed using the uv transilluminator (cleaver scientific Ltd UK).

Appling the agreement test.

We have calculated the agreement rate between the testes applied according to the following relation:

$$\text{Total agreement rate} = \frac{\text{n° of (PI) in both tests + n° of (NI) in both testes}}{\text{Total number of tested isolates}}$$

(PI): positive isolates, (NI): negative isolates

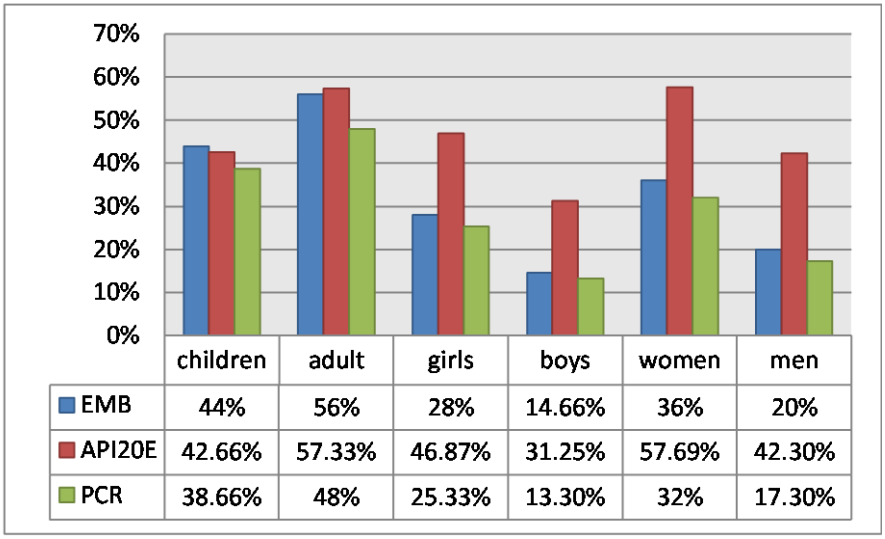
Results:

Identification based on morphological characteristics using EMB agar.

All isolates on EMB selective agar were identified as *E. coli*. This result was identified depending on the distinctive metallic sheen and the Small gloomy of bacterial colonies. Genus of colony was identified based on morphological characteristics without the use of any other confirmation methods. The *E. coli* gives on EMB agar after 16-24 hours incubation in 37°C purple black colonies with distinctive metallic green sheen that is the accredited stander for identification of the *E. coli*. We transplant all isolates on EMB agar for purification and application of comparison.

It should be noted that some of the subculture isolates lost the metallic green sheen after one or two weeks saving in the refrigerator, which was excellent in the previous primary agriculture. The identification results were distributed as follows, 44% (n=33) children. It was distributed between 28% (n=21) girls and 14.66% (n=11) boys. 60.46% (n=26) in adults was distributed between 57.69% (n=15) females and 42.30% (n=11) males (figure 1).

Figure 1: Shows the percentage of the distribution of infection among samples taken by sex and age.



According to results of applying three different methods (EMB, API 20E, PCR) for diagnosis isolates we noticed the same distribution rate, the infection was in women and girls higher than in men and more boys.

Identification based on Biochemical characteristics using API20E set.

Results from the API 20E test showed that 51 isolates out of the 75 earlier identified as *E. coli* using EMB agar gave a positive result as *E. coli* after applying biochemical tests (Table 1).

Table 1: Identification of 24 selected *E. coli* isolates by API 20E, PCR

Isolate reference number	**Identification by EMB**	**Identification by API 20E**	**Identification by PCR**
EC 3	Identified as *E. coli*	*E. coli / salmonella*	+
EC 12	Identified as *E. coli*	*E. coli / salmonella*	+
EC 28	Identified as *E. coli*	*E. coli / salmonella*	+
EC 56	Identified as *E. coli*	*E. coli / salmonella*	+
EC 40	Identified as *E. coli*	*Klebsiella*	+
EC 41	Identified as *E. coli*	*Klebsiella*	+
EC 54	Identified as *E. coli*	*Klebsiella*	+
EC 75	Identified as *E. coli*	*Klebsiella*	+
EC 14	Identified as *E. coli*	*E. coli / citro*	+
EC 17	Identified as *E. coli*	*E. coli / citro*	+
EC 27	Identified as *E. coli*	Out of specification	_
EC5, EC9, EC18, EC22, EC34, EC39, EC44, EC46, EC48.	Identified as *E. coli*	Out of specification	_

24 isolates out of 75 were identified as not *E. coli* using API 20E. 14 isolate out of these 24 were out of specification. After applying PCR the same 24 isolates targeting a fragment of the *E. coli* 16S rRNA gene except tow isolates (EC17 and EC27) they gave no PCR products, were not *E. coli*.

Identification was with various levels of accuracy (Table 2); some isolates has one possibility as *Klebsiella*, or *E. coli* and other isolates has two possibilities:

E. coli/*Salmonella* the (id) 57.4% and (id) 41.9%. Another isolates has two possibilities are: *E. coli* (id) 74.5% and *Citrobacter* (id) 23.1%. It is important to note that some samples had two close possibilities for the same genus:

E. coli (1) the (id) 69.5% and the *E. coli* (2) (id) 30.2%.The rest (fourteen) did not recognize in the index approved by the manufacturer (out of specification). The identification results were distributed as follows, 78.12% (n=32) children. It was distributed between 46.87% (n=15) girls and 31.25% (n=10) boys. 60.46% (n=26) in adults was distributed between 57.69% (n=15) females and 42.30% (n=11) males (Figure 1).

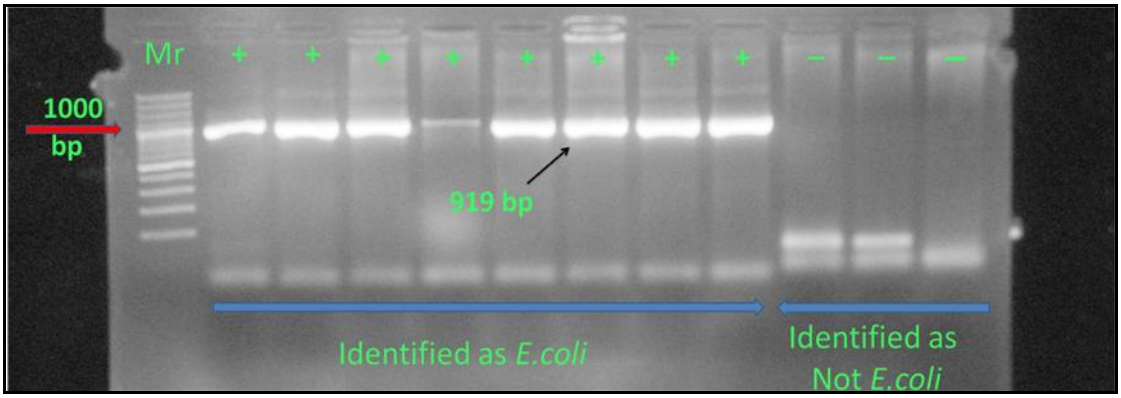
Table 2: Levels of identification for all 75 isolates by API 20E

Level of identification	isolates (n = 75)	
High level of discrimination(90% and above)	32	Identified as *E. coli* (62.7%)
Low level of discrimination(72% and below)	19	Had two possibilities (37.3%)
Out of specification	14	did not recognize in the index
Not identified as *E. coli*	10	as *Klebsiella*, and other species

Molecular identification using PCR.

Identification of isolates by PCR showed that 86.6% (n=65) isolates out of 75 produced the expected PCR products (fragment of 919 bp) (Figure 2). While, 10 isolates were negative (Table 2). The comparison between negative results of biochemical identification using API 20E kit and results of molecular for the same isolates were showed in (Table 3). The results were distributed as follows, 38.66% (n=29) children. It was distributed between 25.33% (n=19) girls and 13.30% (n=10) boys. 48% (n=36) in adults was distributed between 32% (n=24) females and 17.30% (n=12) males (Figure 1).

Figure 2: Agarose gel electrophoresis of amplified DNA products in PCR.



Lane Mr: Molecular size markers, Lane (+): positive isolate, identified as *E. coli* produced the expected PCR products (fragment of 919 bp). Lane (-): negative isolate, not *E. coli*.

Table 3: Negative isolates by identification in PCR

Isolate reference number	Identification by EMB	Identification by API 20E	Identification by PCR
EC 10	Identified as *E. coli*	*E. coli* 1/2	—
EC 17	Identified as *E. coli*	*E. coli* /citro	—
EC 21	Identified as *E. coli*	*E. coli*	—
EC 27	Identified as *E. coli*	out of specification	—
EC 29	Identified as *E. coli*	*E. coli*	—
EC 47	Identified as *E. coli*	*E. coli*	—
EC 57	Identified as *E. coli*	*E. coli*	—
EC 61	Identified as *E. coli*	*E. coli*	—
EC 64	Identified as *E. coli*	*E. coli* 1/2	—
EC 70	Identified as *E. coli*	*E. coli*	—

Identification of isolates by PCR showed that only 10 isolates out of 75 (86.6%) not produced the expected PCR products. These isolates were also successfully identified by API 20E and EMB agar except Tow isolates (EC 17 and 27) were not identified by PCR And were not identified by API20E although.

Appling the agreement test.

The results of our study showed that number of common negative isolates in API 20E and RCR were 2 isolates (EC17, EC27) and 41 common positive isolates in tow testes. The agreement percentage was 57.3%. While the agreement percentage among API 20E, EMB was 68%, and among PCR and EMB was 86.6% (Table 4).

Table 4: Agreement rates among testes using for identification of *E. coli*

Testes	API 20E	PCR	EMB
EMB	68%		
API 20E		57.3%	
PCR			86.6%

Discussion

In this study, 75 samples of UTIs for adults and children in the form of isolates on EMB selective agar were identified as *E. coli* depending on the distinctive metallic sheen and the Small gloomy of bacterial colonies. However, it ais known that some genera such as *Enterobacter* and *Klebsiella* and *Citrobacter* of fermented lactose bacteria may produce such a little sheen on this agar because of its ability to ferment this type of sugar [2, 16]. This explains why some isolates that identified by conventional method are not confirmed as *E. coli* by the biochemical approach.

Furthermore, in this study, we noticed that the colonies lose distinctive metallic sheen or the intensity decreases after saving in 4 °C in the refrigerator for more than two weeks or after cultivation for several consecutive times for purification. Growing colonies on solid media suffer from adjustment phenomena (adaptation), when changing environment from anaerobic to aerobic conditions.

P. R. Lewis, C. N. Hinshelwood, mentioned that Changes in growth rate have been correlated with changes in the reducing power accompanying transitions from one medium to the other or from anaerobic to aerobic conditions and vice versa. Two types of adjustment play a part in the observed phenomena: (a) rapid changes in concentrations of active intermediates, (b) slow adaptive modifications of enzyme systems in response to needs of the cell [18]. Changes in enzyme systems make the colonies lose some of their apparent characteristics as color of the colonies. That may help explain the disappearance of metallic Sheen at replanting on EMB agar for purification, and that some colonies appeared smaller in diameter than its normal size despite it owns all of the other characteristics of *E. coli*. Some isolates obtained in this study show weak sheen. These isolates described as *E. coli* after biochemical diagnosis. Results reported by other study[11], indicating similar finding and this finding might be important for accurate diagnosis of *E. coli*. The rapid commercial identification systems were developed to provide fast and accurate identification of bacteria. The API 20E is a standardized miniaturized system that was developed by Analytab Products Inc. in the 1970s and still in widespread use for the identification of *Enterobacteriaceae* and some other gram negative rods. In previous investigation [20], *E. coli* is characterized by the following: rhamnose Positive - fermentation of sucrose varies from strain to another - negative inositol - produce indole – all strains negative test Fox Proskauer (Asitoin production) - all strains negative urease - negative production of H2S - not dilute Gelatin - negative Citrate, These findings concur with our study.

In this study, our results showed that isolates were identified by API 20E system with various level of accuracy (Table 1). 62.7% (n=32) of *E. coli* isolates were accurately identified. This low accuracy is probably due to incubation for 24 h only. Other study noted that after 24 h the accuracy of API 20E identification was 87.7 %, however after 48 h incubation, they noted an increase to 96.3 % accuracy[21].

Comparison of the results indicates that the injury rate among adults is higher than in children. It was noted that, the injury rate in women more than in men. Besides, they were also in non-adult females more than in non-adults males as indicated in many other studies[6, 26]. Recording positive or negative results using API 20E kit depends on visual observation of the change in color in each interaction. This change in color happen

because of the indicator existence which distinguishes the transformation happening. Sometimes, the change in color is difficult to be noted between the two cases, For example, the change between light red and rose. Other times, invisible errors may occur in laboratory work lead to dryness and evaporation in some wells or contamination or variation in the amount of bacteria added per well. All of this is source of error which may occur in biochemical investigations ways and lead to ambiguous or false results. Similar finding has been reported by [21]. It can be noted from the results of [21, 28, 30] that incorrect identification of *Enterobacteriaecae* by API 20E has been reported due to aberrant reactions by API and/or atypical strains.

For this reason, all tested *E. coli* isolates identified by biochemical reactions methods are confirmed by molecular approach. Molecular diagnostic method is based on the amplification of sequence of 16S rRNA gene by PCR using Thermal circular device.

In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hyper variable regions that can provide species-specific signature sequences useful for identification of bacteria[13, 24]. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification[5]. Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species[19], or even genera[4, 29]. It has also been used to describe new species that have never been successfully cultured[4, 9].

Depending on the PCR, 65 isolates were identified as *E. coli* while the other 10 isolates were negative. The results of the PCR have matched by 95 % with the results of identification by conventional method. 24 negative isolates identified by API 20E are confirmed as *E. coli* by the molecular approach except 2 isolates were negative (not *E. coli*). All of the out of specification isolates except one isolates were *E. coli* based on the PCR. And the differences between results of biochemical and molecular identification may return to the many sources of error which may occur during the applications of API 20E as previously explained, and to the inability of the biochemical testes to identify some isolates which explains the continuous adjustments of API 20E system by the manufacturers. These results consistent with results reported previously[11].

Formerly study has showed that identification of *E. coli* isolates of clinical and environmental origins by rapid commercial systems should be interpreted with care. PCR might be used to further confirm the result of rapid identification systems[1]. In our study, according to the results of agreement test, the agreement percentage among EMB and PCR was 86.6%. Therefore, further confirmation by PCR should be applied after identification by conventional methods.

In conclusion, the conventional method cannot be the only way to give an accurate diagnosis result because it is known that some genera such as *Enterobacter*, *Klebsiella* and *Citrobacter* of fermented lactose bacteria may produce such a little sheen on EMB agar. This explains the misidentification of some isolates as *E. coli* depending on conventional method using this agar. Some isolates identified as *E. coli* using EMB agar gave a negative results after applying biochemical tests and the misidentification rate was 32%. Our results confirmed the Inaccuracy of *E. coli* identification based on her formal characteristics only. After applying PCR, In comparison with the results of API 20E the misidentification rate is 13% because we founded that some of the negative API results were positive after applying PCR. We have noticed that the misidentification rate decreased. The previous work draws attention to the possibility of high error rate happened while the diagnosis of *E. coli* in a conventional and biochemical methods. Therefore, the application of PCR for identification of the genus and even for species is of utmost importance.

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